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Taxol is a new potent chemotherapeutic agent for the treatment of metastatic breast cancer. However, many tumors show partial or complete resistance to taxol therapy. Biological mechanisms modulating taxol response in human cells are mostly unknown. A better understanding of molecular determinants of taxol response may lead to therapeutic strategies designed to prevent or overcome drug resistance.

I am using a methodology based on genetic suppressor elements (GSEs) to identify molecular mechanisms of taxol response. An equal abundance small fragment library in a retroviral vector has been constructed in our laboratory from total human cDNA. I used this library to select GSEs conferring taxol resistance in human cells. After three rounds of infection, selection and insert recovery, putative GSEs were identified. These sequences are undergoing additional testing in human breast carcinoma cells.

A rapid and efficient method for recovery and regeneration of integrated proviruses has been developed in the course of this selection and was shown to accurately maintain the complexity of retroviral population.

I constructed a series of retroviral vectors with various forms of green fluorescent protein as a marker for transduction. Use of these vectors avoids common artifacts associated with other marker genes, and provide sensitive means to monitor the biological activities of transduced sequences. A new library in one of this vectors is now available for future selections on breast carcinoma cells.

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Introduction.

Chemotherapeutic treatment, based on antiestrogens (such as tamoxifen) or cytotoxic chemotherapeutic drugs (doxorubicin, cyclophosphamide, methotrexate, 5-fluorouracyl) has shown limited efficacy in the treatment of breast cancer (1). The major new drug to enter the chemotherapeutic armamentarium for this malignancy in the past decade is taxol, an alkaloid extracted from the bark of *Taxus brevifolia*. In a number of clinical trials taxol-induced response rate in breast cancer varied from 20 to over 60% depending on regimen, dose and prior treatment (2). Objective response was found even in the metastatic cancer resistant to doxorubicin or other means of extensive therapy (2-4). However, a substantial number of tumors demonstrated only a partial response or no response at all. Furthermore, taxol dose escalation is limited by its side effects, primarily myelotoxicity, causing a need for an efficient strategy to protect normal tissues from possible damage. Understanding the molecular determinants of taxol sensitivity or resistance in breast carcinoma cells is likely to result in the development of chemotherapeutic regimens aimed at avoiding or reversing clinical resistance to taxol. In addition, it could lead to the development of strategies to overcome dose limiting side effects of taxol, allowing the improvement of the patients' quality of life and at the same time escalating the therapeutic dose.

The aim of the present study is to identify the genes that determine resistance or sensitivity of human cells to taxol. It is well documented that intracellular accumulation of paclitaxel causes hyperpolymerization of microtubules (5-6), prevents normal cell cycle progression and induces programmed cell death (apoptosis) (7). Known mechanisms of taxol-resistance include drug efflux by P-glycoprotein (Pgp) (5), accumulation of an anti-apoptotic protein Bcl-2 (8) and alterations in tubulin (9-11) that prevent paclitaxel binding. However, the role of Bcl-2 and tubulin alterations in clinical taxol resistance has not been sufficiently documented, and the reported incidence of Pgp positivity in breast carcinomas cannot explain the majority of resistance cases (12).

Our approach to the identification of chemotherapeutic sensitivity genes is based on the isolation of genetic suppressor elements (GSEs), derived from such genes and inducing cellular resistance or sensitivity to the corresponding agents. GSEs are short cDNA fragments that counteract the genes from which they are derived by encoding inhibitory peptides or antisense RNAs (15-19). Our laboratory has previously developed the methodology for GSE selection from retroviral libraries carrying short random fragments of normalized (uniform-abundance) cDNA from mammalian cells and identified several GSEs conferring resistance to anticancer drugs or inducing neoplastic transformation. The same strategy is being used in the present project to isolate GSEs that render human cells resistant to paclitaxel. The cloned GSEs will be used to identify full-length cDNA sequences of the corresponding genes. The genes giving rise to such GSEs would constitute likely determinants of taxol sensitivity in breast carcinoma.

Body.

1. Brief summary of the preliminary data.

A library of randomly oriented short cDNA fragments in retroviral vector pLNCX (20) has been constructed in our laboratory. For a target cell line, I have engineered HT1080 human fibrosarcoma cell line to express the cDNA for murine ecotropic receptor (21). This modification enables us to use very efficient transduction with high titer ecotropic retroviruses that are free of helper virus and not infectious to humans (22). This cell line, HT1080E14, appears to be a suitable model for drug-response in human cells, as it is sensitive to all chemotherapeutic compounds tested, including paclitaxel. A fraction of the library has been introduced into HT1080E14 cells and infected population (~$4x10^6$ independent infectants) was subjected to 2 rounds of taxol treatment. A new method for provirus rescue based on Long and Accurate PCR (23) has been developed in our laboratory (24) and used to transfer the reduced complexity provirus mixtures into fresh populations of HT1080E14 cells (secondary infectants). After another round of provirus transfer and taxol selection, PCR analysis revealed retention of 1-8 individual inserts in each experimental population. Noticeably higher survival of experimental cells as compared to vector infected control, suggested that secondary and tertiary infected populations may in fact be enriched for cells containing active GSEs.

2. Identification of sequences enriched in the course of taxol selection.

I have isolated a number of individual cDNA fragments enriched in separate populations of secondary and tertiary infectants. These fragments were cloned into LNCX and introduced into naive HT1080E14 cells via retroviral transduction. Pure populations containing putative GSEs were obtained by subjecting infectants to G418 selection. Subsequently, G418 selected cells were treated with various concentrations of taxol and survival on experimental plates was compared to that among vector-infected cells. Some experimental populations appeared to have increased resistance to paclitaxel (Fig. 1), suggesting the presence of biologically active inserts. Putative GSEs were sequenced and compared to the known sequences deposited in GeneBank. Among these fragments we focused our attention on putative GSE 2a1. This putative GSE encompasses an antisense oriented fragment of the cDNA for human cytochrome c oxidase subunit III (cox3), a mitochondrially encoded protein involved in oxidative phosphorylation. Interestingly, a similar but not identical cDNA fragment was later isolated in our laboratory as a putative GSE in the course of selection for resistance to DNA replication inhibitor aphidicolin. Position of both fragments within the cox3 sequence is shown on Figure 2. Enrichment of cox3 derived sequences in independent selections may suggest that compounds with such dissimilar primary targets as aphidicolin and taxol share some common mechanisms of cell killing. In fact, cox3 involvement in the programmed cell death is consistent with recent findings that changes in

cytochrome c status are a hallmark of apoptosis (25-26). Our laboratory is currently investigating cellular phenotype associated with cox3-derived GSE expression, including alterations in mitochondrial functions and responses to various cytotoxic and cytostatic agents.

3. Development of a new method for recovery and regeneration of integrated retroviruses (24, reprint attached).

For the transfer of proviral populations from selected to naïve cells for consecutive rounds of selections we relied on the method of rapid regeneration and recovery of integrated proviruses, that was recently developed in our laboratory. The method is based on PCR amplification of complete provirus followed by transfection of amplified products into packaging cells. To validate this approach we demonstrated that at every step of this protocol complexity of a mixed viral population is sufficiently maintained. We were able to show that individual cDNA fragments enriched after taxol selection retained their relative frequencies upon transfer into fresh HT1080E14 cells. In addition, similar amount of infectious virus was produced upon transfection of packaging cells with 1 μg of vector, either in the form of supercoiled plasmid or linear PCR product. Furthermore, an intermediate step of gel purification followed by more rounds of amplification can be used to enhance sensitivity and specificity of the reaction, as well as to generate preparative quantity of complete provirus from minimal amounts of starting material without accumulation of shorter products. The entire procedure, from integrated proviruses in genomic DNA to generation of new infectants, takes as little as three days.

4. Construction of novel retroviral vectors: GFP as a marker gene.

a. Vectors for constitutive expression.

Despite some encouraging results in GSE selections we often encountered substantial variability in resistance profiles between cell populations harboring the same exact cDNA fragment. Sometimes, while secondary and tertiary infectants appear to be more resistant than respective vector-infected controls, no difference was apparent in individual testing of certain enriched sequences (27). A possible explanation for these phenomena comes from studies of gene expression from bicistronic vectors conducted in our laboratory (28). In the presence of G418 selection there appears to be a selective pressure in favor of cell clones that may have increased neo gene expression at the expense of silencing a cotransduced transcriptional unit. Ultimately, selected population may become dominated by cells that no longer express a gene of interest. Taking into consideration this phenomenon, as well as the length of G418 selection and the inevitable stress that it applies even onto neo-expressing cells, we decided to redesign our retroviral vectors. We have chosen to substitute a drug-resistance marker (e.g. neo in LNCX) for *Aquarea victoria* gene for the green fluorescent protein (GFP) (29). This naturally fluorescent protein

retains its fluorescence (peak excitation at 376nm, emission - at 510nm) when expressed in a heterologous host. Detection of GFP fluorescence is non-invasive and can be performed on living cells of various origins. However, my attempts to detect fluorescence of HT1080E14 cells infected with LNCGFPwt (wild type GFP in LNCX vector) were unsuccessful. Detection equipment available in our laboratory is optimized for fluorescein isothiocyanate (FITC) that has a similar emission spectrum to wild type GFP, but significantly different excitation maximum (~490 nm). Thus, I hypothesized that S65T mutation in GFP, that shifts the excitation peak to 490nm (30), may improve GFP detection in our conditions. To verify this prediction I introduced "red-shifted" GFP S65T into LNCX to construct LNCR vector (Fig.3). LNCR infected cells were readily detected by flow cytometry. I considered it important to construct a vector of "LTR-GFP-CMV-cloning sites-LTR" structure, because this design places a gene of interest under the control of a potent cytomegalovirus promoter. However, when I substituted neo in LNCX for GFPS65T (LRCX vector in Fig.3), GFP expression from a single copy provirus appeared to be insufficient for unambiguous identification of infectants. I have then used "Green Lantern" GFP from Gibco BRL that has been engineered to alter codons in the GFP S65T sequence that are unfavorable for mammalian translation machinery (31). When cloned in LNCX , "Green Lantern" GFP produced 15-20 fold higher level of fluorescence than GFPS65T (Fig. 3). To further facilitate GFP expression I used a modified Moloney Murine Leukemia Virus LTR that harbors an extra binding site for Sp1 transcription factor. This modification not only increases the level of expression in the common host cell lines, but also prevents LTR silencing in a number of cell types normally non-permissive for Moloney LTR expression (32). The resulting LmGCX vector (Fig. 3) conferred clearly detectable fluorescence in HT1080E14 cells as well as in a number of other cell lines, including MCF7 breast carcinoma. LmGCX has been chosen to construct a new normalized total cDNA fragment library. This project has recently been accomplished in our laboratory using MCF7 breast carcinoma cells as an mRNA source. The more recent GFP variant, "Enhanced GFP" (EGFP from Clontech), combines complete "humanization" of codons with S65T mutation and other changes that decrease GFP aggregation and improve folding and fluorescence (33). I used EGFP to construct LXSE and LmECX vectors (Fig 3). Both of them render higher fluorescence than LmGCX while providing a choice of MoMuSV LTR or CMV promoters to express a sequence of interest. LXSE and LmECX are now our vectors of choice for easy identification of infectants and constitutively high expression of inserts.

Unlike colony formation assays, flow cytometric analysis of treated cells requires minimal time, allows simultaneous analysis and recovery of specific cell groups, and has the power of analyzing hundreds of thousands cells at once to validate statistical significance of even small changes in population structure. I was able to verify that GFP expression is non-detrimental to infected cells and the dynamics of GFP-positive subpopulation is a sensitive indicator for biological effects of cotransduced sequences. Upon LNCG infection the fraction of positive cells remained essentially unchanged for almost three months of continuous

culture without selection. In a separate experiment, the fraction of LXSE infected cells remained constant in non-selected populations for 16 days, while in parallel cultures infected with LWTRSE (p53 tumor suppressor cDNA in LXSE) the fraction of positive cells dropped from seventeen to two percent over the same period of time. I have cloned in LmECX putative GSEs enriched upon taxol selection. This will allow me to assay phenotypic effect of these sequences by measuring the fraction of GFP positive cells before and after taxol treatment. Moreover, I was able to show that fluorescent signal detectable by a multi-well fluorescence reader is directly proportional (r^2=0.95) to the fraction of GFP-positive cells in a mixed population. This observation will allow me to rapidly monitor changes in the structures of multiple populations at one time, or to identify and recover populations transduced at similar rates for use as mutual controls for drug-resistance studies.

b. Inducible vectors.

For the purposes of this project an ability to regulate expression of an insert is highly desirable. This would allow us to identify GSEs that in the absence of selection may have some detrimental effect for a cell. Also, this would provide a very precise control for the biological activity of individual GSEs, since exactly the same infected population may be split in two parts and analyzed with an insert "ON" and "OFF". Considering the benefits of GFP as a marker, we redesigned our IPTG-regulated vectors (34) to substitute *neo* for the "Green Lantern" GFP. Three vectors of this type were made, (Fig.3) that differ in the strength of an internal regulated promoter. Since GFP transcription runs in an antisense orientation relative to the inducible promoter, we are able to monitor IPTG-mediated regulation by the changes in the fluorescence (Fig.4). The uniqueness of this system is that while GFP is used to judge the activity of an internal promoter, this promoter does not have to be turned "ON" for detection of infectants. I am planing to use these vectors for the future testing of individual putative GSEs that demonstrate weak or highly variable phenotypes when expressed constitutively.

c. Methods for identification of multiply infected cells.

We have obtained indications from the experience of other GSE selections, that maximal biological effect of certain sequences is likely to be manifested when a suitable combination of GSEs is present within one cell (27). This possibility implies a need to identify cells that carry more than one integrated provirus. Using green fluorescent protein as a marker gene we developed three strategies to achieve this goal. First, fluorescent signals yielded by GFPS65T and EGFP are so different in intensity, that cells expressing these GFP variants formed two separate positive populations distinguishable by flow cytometric analysis (Fig 5). This observation allows consecutive introduction of two similar constructs: first, the one with a modestly fluorescent form of GFP (e.g. GFPS65T), then, the one with a brighter form (e.g. "Green Lantern" or

EGFP). Transduced population can be collected after each step of infection by fluorescence activated cell sorting.

An alternative approach is to use spectrally distinct GFP variants. We constructed vectors with Enhanced Blue Fluorescent Protein (EBFP from Clontech) that has both emission and excitation spectra (excitation peak at ~360 nm, emission - at ~450 nm) distinct from "red-shifted" GFP variants. Cells carring EBFP (e.g. in the context of LXSB vector, Fig.3) are distinguishable from non-infected or EGFP-infected cells by fluorescent microscopy and flow cytometry. We are currently refining our techniques for specific identification of EGFP-EBFP double expressing cells.

The third approach is based on prediction that cells harboring more copies of GFP-expressing constructs will manifest higher level of fluorescence. We proved this prediction by Southern analysis of single cell subclones obtained from cells within different areas of positive peak (Fig.6). In fact, by collecting only the brightest infectants we were able to select specifically multiply infected cells.

Conclusions.

Several rounds of taxol selection led to enrichment of certain sequences that are likely to represent biologically active genetic suppressor elements. In particular, a fragment derived from human cox3 gene (GSE2a1) attracted our closest attention, as a homologous sequence has been identified in the course of selection to another cytotoxic compound (aphidicolin). Possible mechanisms of action of GSE2a1 are currently being investigated. The initial selection has been carried out on human fibrosarcoma HT1080 cell line and, according to the original plan, I am now in the process of testing the biological activities of putative GSEs in MCF7 breast carcinoma cells.

In the course of this selection we improved the procedure for the transfer of libraries in the form of integrated proviruses into fresh host cells for subsequent rounds of selection. The new method is fast, efficient and accurate, and may be suitable not only for expression cloning in retroviral vectors, but also for the analysis of natural retroviral populations.

In the course of this and other GSE selections conducted in our laboratory we have encountered a need to improve our current vectors. I redesigned common retroviral vectors to include genes for various forms of the green fluorescent protein as a marker gene. Use of GFP as a marker allows us to identify, monitor and select cells as early as two days post infection without using any toxic compounds and costly reagents. Multiply infected cells can be obtained by collecting the brightest cells from transduced populations. We also can select superinfected cells using consecutive infections with vectors caring GFP forms that differ in spectral properties or intensity of fluorescence. While GFP itself does not effect the vital properties of human cells, the dynamics of GFP-positive population is a sensitive indicator for the biological effects of cotransduced gene. I am planning to use this observation as a sensitive test for the participation of putative GSE in drug response. Moreover, the possibility to

measure the presence of GFP-positive cells in a multi-well format will help me to accelerate testing of multiple individual elements. We have also used GFP as a marker in the design of new IPTG-inducible vectors. Regulation of GSE expression should allow me to verify biological activities of those sequences that may have a detrimental effect in the absence of selection.

GFP-containing vectors are our vectors of choice for future selections and a new library in one of these vectors (LmGCX) has been constructed in our laboratory. This uniform abundance small fragment cDNA library was derived from MCF7 mRNA. Such library source should ensure representation of genes that may be involved in taxol response specifically in breast cancer. Also, MCF7 cells that express murine ecotropic receptor were engineered in our laboratory. The original goal of this project was to perform GSE selection and characterization in HT1080E14 human fibrosarcoma cells and subsequently to test biologically active elements on breast carcinoma cell lines. However, availability of the new library and a well infectible MCF7 derivative will allow us to use this library-host combination directly in future selections.

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Appendix.

Figure 1. Enhanced taxol resistance of GSE-containing cells.
BOSC23 packaging cells were transfected with pLNCX, pLNC2A1, pLNC302 plasmids. One and 2 days post transfection viral supernatants were used to infect HT1080E14 cells. Five days later cells were plated in six well plates, 10^4 cells per well. The next day taxol (0, 2, 4, 6, 8, 10, 12 ng/ml) was put on the cells. After two days of continuous treatment cells were transferred into fresh medium and allowed to grow. Methylene blue staining was done three (panel A) or six (panel B) days later. The dye was extracted with 1M HCl (0.5 ml per well) and absorption was read at 600 nm. At 12 ng/ml of taxol no colony formation was observed among LNCX infected cells, while few colonies began to appear among cells infected with LNC2A1 and LNC302. Infection efficiency (>90% for each of the infected populations) was estimated by plating an aliquot of cells for colony formation in the presence and in the absence of G418. pLNC2A1 and pLNC302 are pLNCX derivatives containing GSE2a1 (antisense fragment of cox3 gene) and GSE302 (fragment of an unknown gene homologous to catepsin L) respectively.

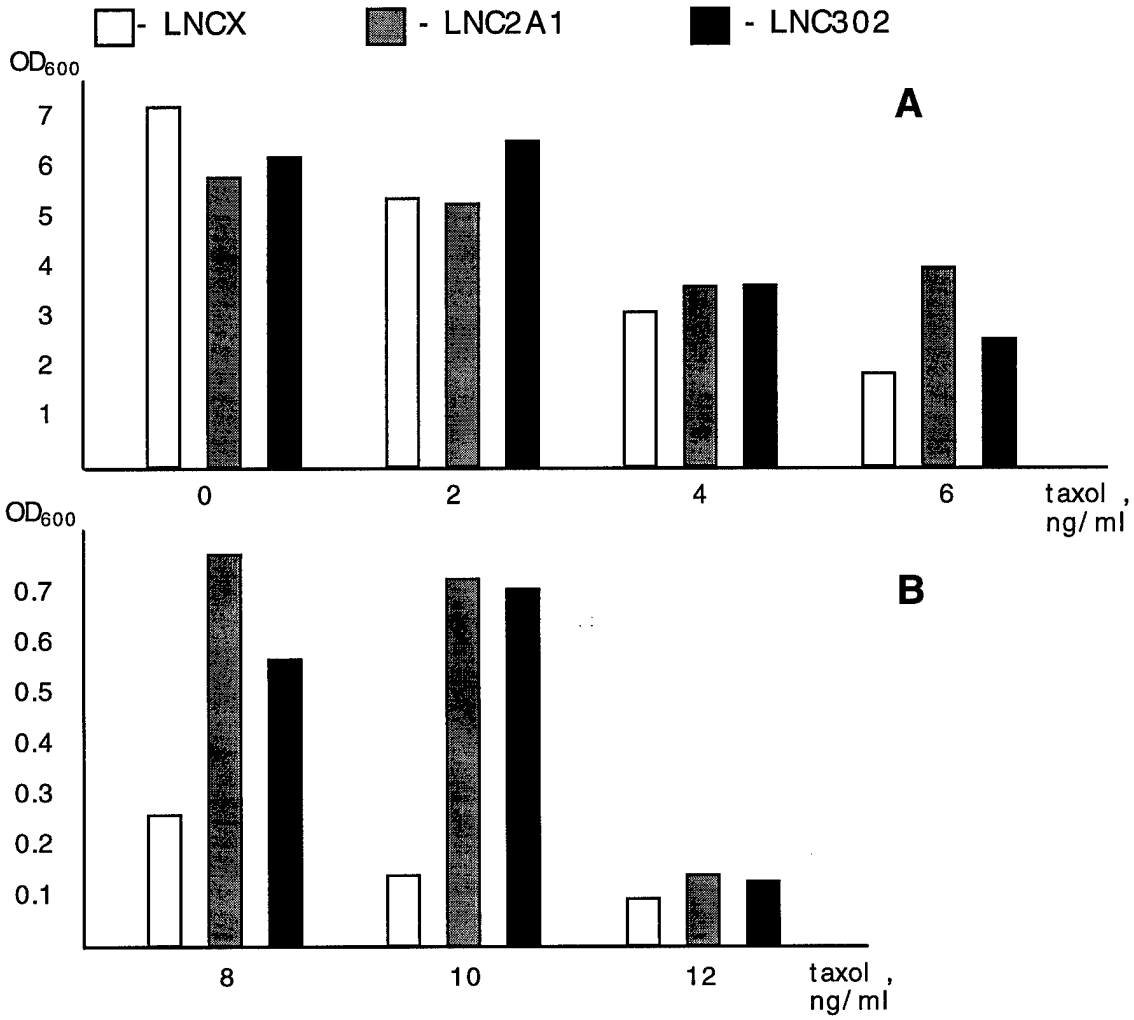
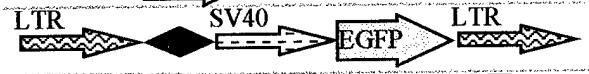


Figure 2. Sequence of human Cox3 gene.
A fragment complimentary to aphidicolin-selected GSE is shown in bold, a fragment complimentary to taxol-selected GSE is underlined.

ATGACCCACCAATCACATGCCTATCATATAGTAAA**ACCCAGCCCATGA<u>CCC</u>**
<u>CTAACAGGGGCCCTCTCAGCCCTCCTAATGACCTCCGGCCTAGCCATGTGA</u>
<u>TTTCACTTCCACTCCATAACGCTCCTCATACTAGGCCTACTAACCAACACAC</u>
<u>TAACCATATACCAAT</u>GATGGCGCGATGTAACACGAGAAAGCACATACCAAG
GCCACCACACACCACCTGTCCAAAAAGGCCTTCGATACGGGATAATCCTAT
TTATTACCTCAGAAGTTTTTTTCTTCGCAGGATTTTTCTGAGCCTTTTACCAC
TCCAGCCTAGCCCCTACCCCCCAATTAGGAGGGCACTGGCCCCGAACAGG
CATCACCCCGCTAAATCCCCTAGAAGTCCCACTCCTAAACACATCCGTATTA
CTCGCATCAGGAGTATCAATCACCTGAGCTCACCATAGTCTAATAGAAAACA
ACCGAAACCAAATAATTCAAGCACTGCTTATTACAATTTTACTGGGTCTCTAT
TTTACCCTCCTACAAGCCTCAGAGTACTTCGAGTCTCCCTTCACCATTTCCG
ACGGCATCTACGGCTCAACATTTTTTGTAGCCACAGGCTTCCACGGACTTC
ACGTCATTATTGGCTCAACTTTCCTCACTATCTGCTTCATCCGCCAACTAAT
ATTTCACTTTACATCCAAACATCACTTTGGCTTCGAAGCCGCCGCCTGATAC
TGGCATTTTGTAGATGTGGTTTGACTATTTCTGTATGTCTCCATCTATTGATG
AGGGTCT

Figure 3. GFP-containing retroviral vectors.
Vectors are shown as integrated proviruses. Relative fluorescence (RF) was calculated as the ratio of median fluorescent signal of infected cells to that of non-infected control. Cell analysis was done using Becton Dickinson FACSsort with argon excitation and GFP detection in FL1 (BP530/30). EBFP is not fluorescent under these conditions, but was clearly detectable when appropriate optics were used. RF for inducible constructs was measured in the absence of inducing agent.

VECTOR STRUCTURE	NAME	RF in HT1080
	LNCR	10-20
	LRCX	2-3
	LNCG	100-200
	LmGCX	8-10
	LXSE	20-40
	LmECX	10-15
	LGXRO2	20
	LGXCO3	8-10
	LGXCO4	8-10
	LXSB	N.A.

Components of the retroviral vectors

	Moloney murine leukemia virus Long Terminal Repeat (LTR)
	Moloney murine leukemia virus LTR with an extra Sp1 site .
	Simian Virus 40 promoter and enhancer.
	Human cytomegalovirus immediate early promoter (CMV).
	Fragment containing modified CMV and *lac*-operators from LNXCO4 (34).
	Fragment containing modified CMV and *lac*-operators from LNXCO3 (34).
	Fragment containing modified RSV LTR and *lac*-operators from LNXRO2 (34).
	Transcription termination/polyadenylation signal from HSV TK.
	A gene for neomycin phosphotransferase II.
	S65T mutant of GFP.
	"Green Lantern" GFP.
	"Enhanced GFP".
	"Enhanced Blue Fluorescent Protein".
	Recommended cloning sites. Vary from vector to vector.

Figure 4. Transcriptional regulation of inducible promoter in IPTG-regulated vectors inversely correlates with changes in GFP expression.

HT1080-3'SS6 cells were infected with LGXCO3 virus, GFP-positive subpopulation was collected and split into two parts. One part was cultivated in the presence, the other - in the absence of IPTG. The histogram plot shows the fluorescent profiles of induced (solid line) and non-induced (dotted line) GFP-positive cells compared to the non-infected control (thin line). Induction of the modified CMV promoter was independently verified by quantitative RT-PCR assay (not shown).

Conditions of flow cytometry were as follows. Cell were trypsinized, washed with PBS and resuspended in PBS at ~10^6 cells/ml. Propidium iodide (PI) was added to 1μg/ml. Fluorophores were excited with an argon laser (488nm) and GFP fluorescence was detected using BP530/30 filter in FL1. Only viable (PI-negative) cells were analyzed.

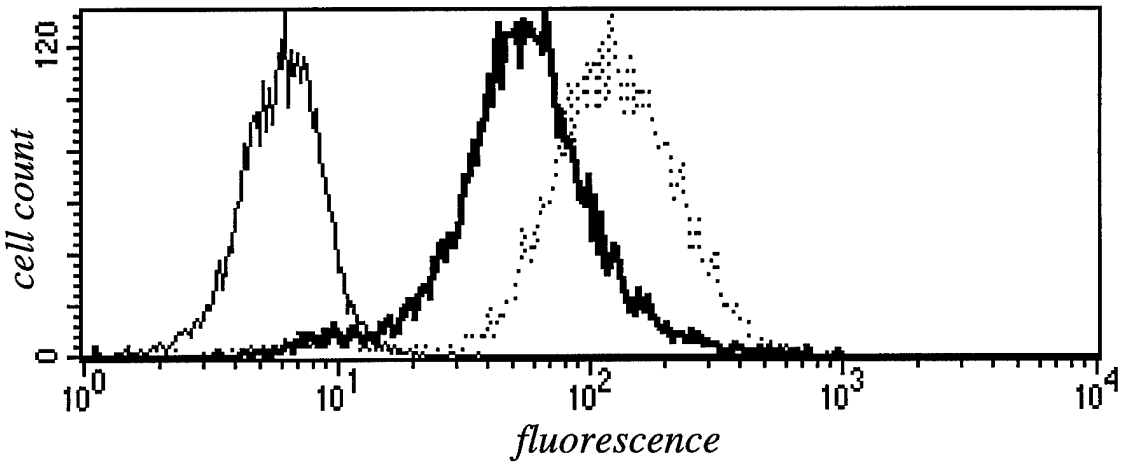
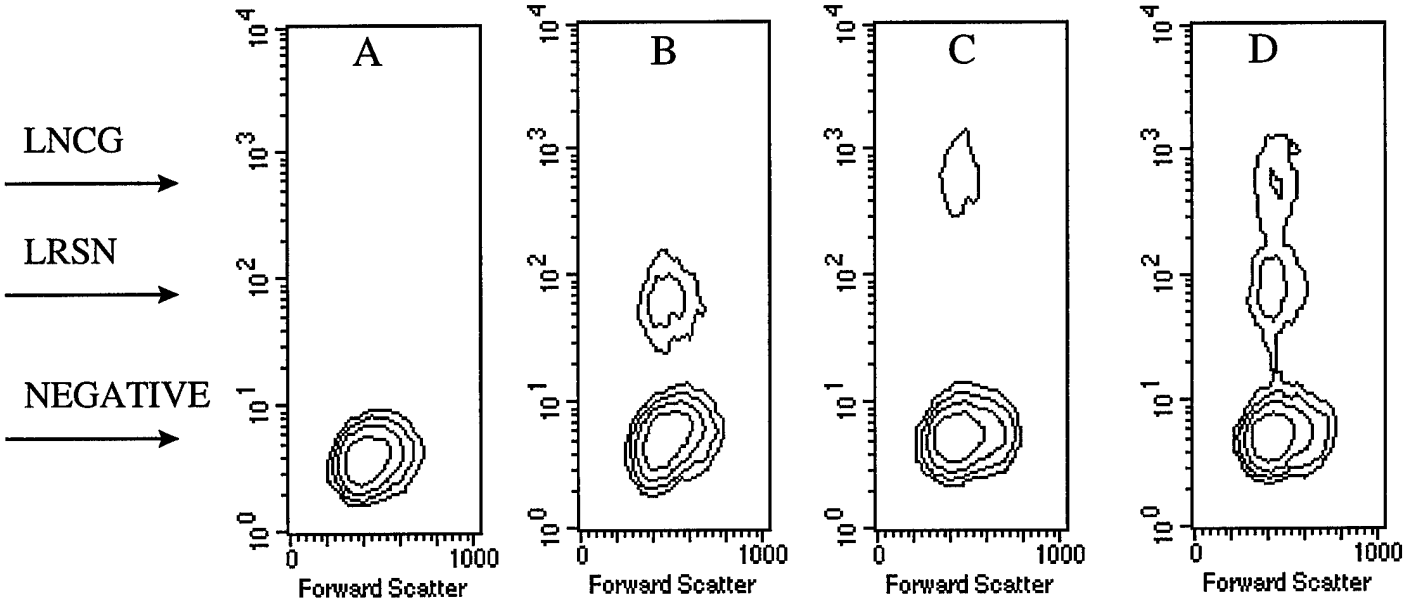


Figure 5. Simultaneous detection of two GFP-containing vectors.

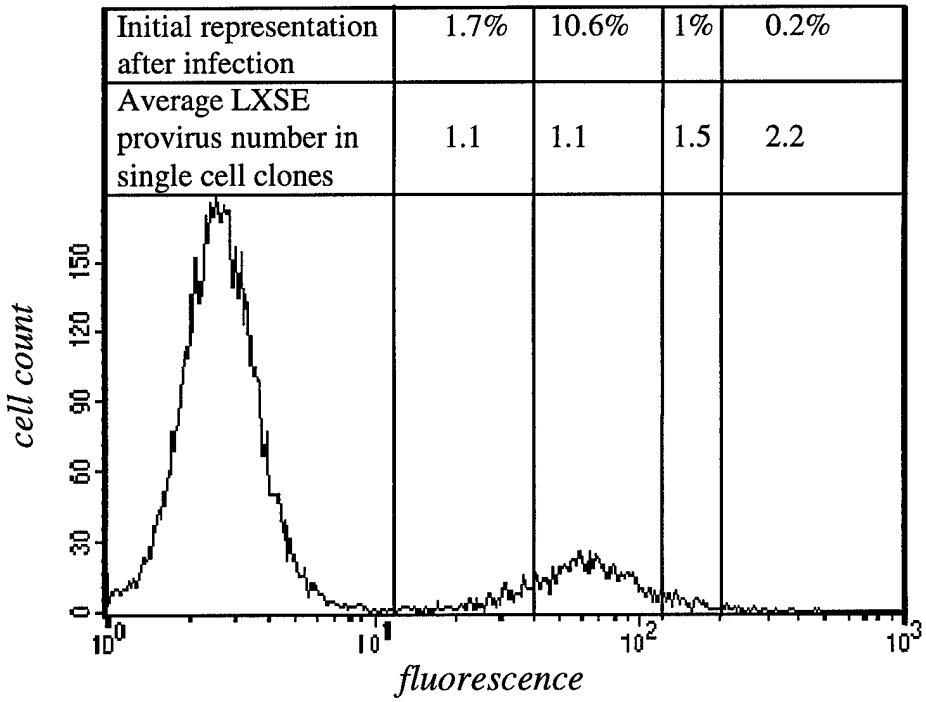
Human fibrosarcoma HT1080-3'SS6 cells were infected with individual LRSN or LNCG vectors (panels B and C) or consecutively with both vectors (panel D) and compared to the mock-infected control (panel A) two days after the second infection, using FACSort flowcytometer (Becton Dickinson) with argon laser excitation. GFP fluorescence was detected in FL1 channel using BP530/30 filter. Only viable (propidium iodide excluding) cells were included in the analysis. The analyzed cell populations are shown on the contour plots of fluorescence (Y axis) versus forward scatter (X axis) with 1 smoothing iteration and 3.2% threshold.



1ST INFECTION	MOCK	MOCK	LRSN	LRSN
2ND INFECTION	MOCK	LNCG	MOCK	LNCG

Figure 6. Brighter LXSE infectants contain higher number of integrated proviruses.

HT1080-3'SS6 cells were infected with LXSE virus from Bosc23 packaging cells. Individual cells were selected from four parts of the positive peak. DNA from the established single cell subclones was extracted and the copy number of integrated proviruses was estimated upon Southern hybridization with an EGFP-specific probe. Initial representation after infection and estimated average copy number of integrated proviruses are shown above corresponding areas of the fluorescence histogram for the infected population on the day of sorting.



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Efficient recovery and regeneration of integrated retroviruses

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ABSTRACT

We report a rapid and efficient PCR-based rescue procedure for integrated recombinant retroviruses. Full-length proviral DNA is amplified by long-range PCR using a pair of primers derived from the long terminal repeats (LTR), and virus is regenerated by transfecting retrovirus-packaging cells with the PCR-derived provirus. The viral yield from the PCR product is similar to that from the retroviral plasmid vector, and the representation of different inserts is accurately maintained in the recovered retroviral population. This procedure is suitable for expression cloning from retroviral libraries and should be applicable to the analysis of natural retrovirus populations.

Retroviral vectors provide one of the most efficient means for gene transfer in mammalian cells. Among other applications, such vectors are used to construct high-complexity libraries for expression cloning of genes (1–3) or genetic suppressor elements (GSEs) (4–6). An integral step in expression cloning is vector recovery from the cells selected for the phenotype of interest; the recovery should be efficient and should adequately reproduce the complexity of the insert sequences present in the selected cells. The usual procedure for the recovery of integrated retroviral vectors involves PCR amplification of inserts from integrated proviruses, which is followed by a labor-intensive step of recloning the PCR products (4). The alternative biological rescue procedures (superinfection with helper virus or fusion with retrovirus-packaging cells) are apt to change the representation of different inserts in the rescued virus population due to differences in virus production by different cells. We have now developed a protocol for rapid and efficient recovery and regeneration of integrated proviruses, which does not require cloning and maintains sequence representation in the recovered virus population. This protocol uses long-range PCR (7,8) to recover functional proviral DNA, which is then used to generate retroviral particles by transient transfection of retrovirus-packaging cells.

The recovery protocol has been developed for the most commonly used type of retroviral vectors based on Moloney murine leukemia/sarcoma viruses and typified by LNCX (9). As illustrated in Figure 1A, the pLNCX plasmid vector contains different 5′ and 3′ LTR sequences; after reverse transcription, both LTR of the integrated provirus acquire the U3 region from the 3′ LTR and the U5 region from the 5′ LTR (10). To amplify the full-length proviral DNA, we have used a sense-oriented (LTRs) primer based on the U3 sequence of the 3′ LTR (5′-AATGAAAGACCCCACCTGTAGGTTT-GGCAAGCTAG-3′) and an antisense-oriented (LTRas) primer from the U5 region of the 5′ LTR (5′-CAAATGAAAGACCCCCG-TCGTGGGTAGTCAATCAC-3′). Genomic DNA was extracted from retrovirus-transduced cells using Qiagen Blood and Cell Culture DNA kit (high molecular weight and purity of the DNA preparation are critical for the procedure).

Each PCR reaction (50 µl) contained 0.2 mM each of the four dNTPs, 0.5 µg each of LTRs and LTRas primers and 0.5 µg genomic DNA template. In earlier experiments, PCR was carried out in Taq extender buffer (Stratagene) using 10 U *Taq* DNA polymerase (Promega) and 10 U Taq Extender (Stratagene) per tube. In more recent experiments, we have utilized instead TaqPlus Long low-salt buffer and 5 U of TaqPlus Long polymerase mixture (Stratagene); these conditions provided higher and more reproducible PCR yield. PCR was performed in a Perkin Elmer Cetus thermocycler under the following conditions: 3 min at 94°C; 27 cycles of 1 min at 94°C, 1 min at 65°C, and 2.25 min at 72°C; followed by 5 min at 72°C. Figure 1B (lane P) shows the result of a reaction carried out on the DNA from human HT1080 cells transduced with LNCX. The reaction yields two products, a short 0.7 kb band corresponding to the LTR, and a long 4.1 kb band corresponding to the full-length provirus. These conditions have been successfully used to amplify proviral DNA for LNCX or LXSN (9) based vectors (tested with inserts of up to 1.7 kb in the cloning site) in several different types of human cells. In contrast, the same PCR conditions applied to murine cells transduced with the same vectors yielded almost no full-length proviral DNA detectable by ethidium bromide staining, due to cross-reactivity of the LTRs and LTRas primers with LTR of endogenous murine retroviruses. Amplification of proviral DNA from murine cells was made possible, however, by carrying out a second round of PCR on the provirus-size DNA which was gel-purified (without ethidium bromide staining) after the first round of PCR. This is illustrated in Figure 1C, where genomic DNA template was isolated from murine NIH 3T3 cells that were infected with retroviral vector LRSN, which carries an S65T mutant form of the green fluorescent protein (GFP) (11) in the LXSN vector. After the first round of PCR, 15 µl of the reaction were used for electrophoresis in a 1% agarose gel, and DNA was extracted from the region of the gel corresponding to 3.5–4.5 kb, using QIAquick Gel Extraction Kit (Qiagen). 1/3000 of the recovered DNA was used

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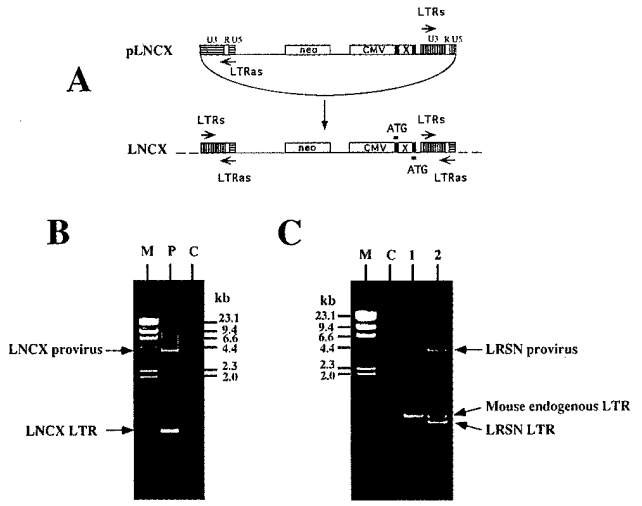

Figure 1. (**A**) Map of LNCX retroviral vector in the form of a plasmid (pLNCX) or integrated provirus. neo, neomycin phosphotransferase gene; CMV, cytomegalovirus promoter; X, insert in the cloning site of the vector. U3, R and U5 indicate the different LTR regions (10). Positions of the primers used for PCR are indicated (LTRs, LTRas, ATG). The 5′ LTR of pLNCX (horizontal stripes) is derived from Moloney murine sarcoma virus, and the 3′ LTR (vertical stripes) from Moloney murine leukemia virus; the origin of the LTR sequences in the provirus is indicated by the corresponding stripes. (**B**) Electrophoretic analysis of a long-range PCR of the DNA from HT1080 cells transduced with the LNCX vector (1% agarose gel). M, size markers (*Hin*dIII-digested λ DNA); P, PCR reaction; C, negative PCR control (no DNA template). (**C**) Electrophoretic analysis of a second round of long-range PCR (see text) of the DNA from NIH 3T3 cells that were uninfected (lane 1) or infected with the LRSN vector (lane 2). Lanes M and C are as in (B).

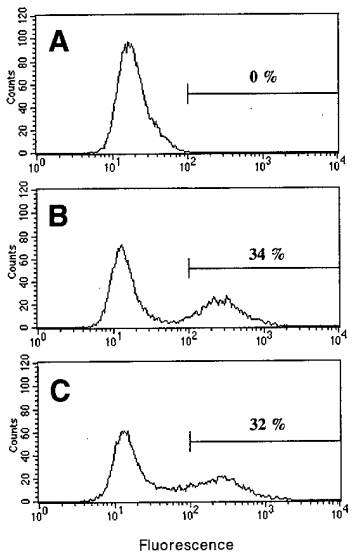

Figure 2. Flow cytometric profiles of HT1080 cells, uninfected (**A**) or infected with retrovirus produced by BOSC23 packaging cells that had been transfected with 1 μg of LRSN supercoiled plasmid vector (**B**) or 1 μg of the PCR-derived LRSN provirus (**C**). 5×10^5 cells were suspended in phosphate buffered saline containing 1 μg/ml propidium iodide (PI). Cells were analyzed with FACSort (Becton-Dickinson) using argon laser excitation (488 nm). PI fluorescence was detected in FL3 emission channel (650LP filter); GFP fluorescence of PI-negative (living) cells was monitored using FL1 emission channel (530/30 BP filter). The data were collected on log scale.

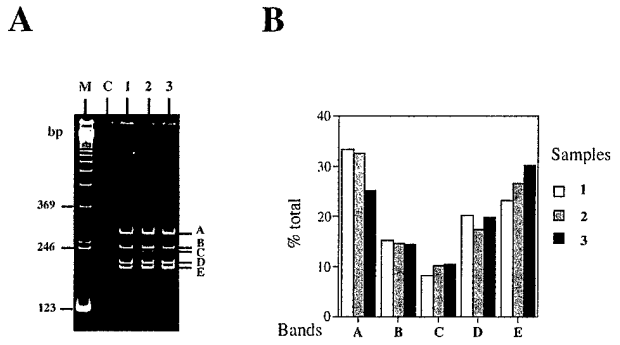

Figure 3. Short-range PCR analysis of representation of different-size inserts in a population of LNCX-derived proviruses. The following templates were used for PCR: genomic DNA of HT1080 cells carrying the integrated provirus population (sample 1), proviral DNA recovered from the same cells by long-range PCR (sample 2), genomic DNA from HT1080 cells transduced with the rescued provirus population (sample 3). Five distinct inserts are designated A–E. (**A**) Electrophoretic analysis of the PCR products in 6% polyacrylamide gel. M, size standards (123 bp ladder); C, negative PCR control (no DNA template). (**B**) Relative intensity of bands A–E in samples 1–3, as measured in ethidium bromide-stained polyacrylamide gel using ISO 1000 gel imaging system (Alpha Innotech). The intensity of each band is represented as the percentage of total intensity of all five bands for a given sample.

for the second round of PCR under the same conditions. This PCR, when carried out on the DNA from uninfected NIH 3T3 cells, yielded a single band presumably corresponding to the endogenous retrovirus LTR, traces of which remained in the gel-purified sample. In contrast, genomic DNA from LRSN-infected cells yielded bands corresponding to the endogenous and vector-derived LTR, as well as a full-length 4.1 kb LRSN provirus (lane 2).

PCR-amplified proviral DNA (combined with salmon sperm carrier DNA to a total of 15 μg) was used to transfect BOSC 23 ecotropic retrovirus-packaging cells (12); the transfection and subsequent infection of recipient cells were carried out as previously described (13). In some experiments, PCR-amplified LNCX-based provirus, recovered from HT1080 cells by a single round of PCR, was purified using QIAquick PCR Purification Kit (Qiagen) and products of one to five PCR reactions were used for transfection. Infected cells were obtained under these conditions, but at a relatively low (<3%) rate. To maximize the viral yield from the provirus derived by a single round of PCR, we gel-purified proviral DNA from a mixture of 20 PCR reactions prior to transfection (without ethidium bromide staining). The efficiency of infection with the LRSN virus recovered under these conditions and the ability of this virus to express functional GFP and Neo proteins were evaluated either by the percentage of fluorescent cells expressing GFP (as measured 3 days after infection) or by the formation of G418-resistant colonies. Figure 2 shows the fluorescence profiles of HT1080 cells (expressing the murine ecotropic receptor, 14) that were either uninfected or infected with retrovirus produced by BOSC23 cells after transfection with 1 μg of LRSN plasmid DNA or the same amount of gel-purified LRSN proviral DNA. The percentage of cells infected with the plasmid-derived virus was 34% by fluorescence and 30% by G418 resistance, while the corresponding values for the recovered virus were 32 and 46%, indicating that the PCR-generated linear provirus was transcribed in BOSC23 cells as efficiently as the supercoiled plasmid. In the case of LRSN recovered from murine

NIH 3T3 cells by two rounds of PCR, the product of a single PCR reaction (purified using QIAquick PCR Purification Kit) yielded the infection rate of 7.6% by fluorescence and 10% by G418 resistance (data not shown).

The maintenance of sequence representation in the recovered retrovirus population is illustrated by an experiment carried out on HT1080 cells that were infected with a normalized cDNA fragment library in the LNCX vector and selected for resistance to taxol (E.S.K., unpublished). The cDNA inserts from retroviral vectors integrated in this subpopulation were amplified by PCR using a primer (ATG) corresponding to the adaptor sequence flanking the inserts (5). Figure 3A shows electrophoretic analysis of PCR products amplified directly from genomic DNA, from full-length proviral DNA recovered by long-range PCR, or from genomic DNA of HT1080 cells infected with the recovered provirus and analyzed 3 days after infection. Each lane contains five distinct bands; their relative intensity is shown in Figure 3B. The representation of different bands is very similar in all three lanes, indicating that sequence representation in this relatively simple population has been maintained throughout the procedure.

In summary, the described provirus recovery protocol is rapid (3 days from genomic DNA extraction to the generation of infectious virus), effficient, and capable of maintaining sequence representation in the retroviral population. This protocol should be useful not only for expression cloning in retroviral vectors but also for functional analysis of sequence variability of full-length genomes in naturally occurring 'quasispecies' of different retroviruses (including HIV).

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